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Specific Binding of Human Immunodeficiency Virus Type 1 *gag* Polyprotein and Nucleocapsid Protein to Viral RNAs Detected by RNA Mobility Shift Assays

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Packaging of retroviral genomic RNA during virion assembly is thought to be mediated by specific interactions between the *gag* polyprotein and RNA sequences (often termed the Ψ or E region) near the 5′ end of the genome. For many retroviruses, including human immunodeficiency virus type 1 (HIV-1), the portions of the *gag* protein and the RNA that are required for this interaction remain poorly defined. We have used an RNA gel mobility shift assay to measure the in vitro binding of purified glutathione *S*-transferase–HIV-1 *gag* fusion proteins to RNA riboprobes. Both the complete *gag* polyprotein and the nucleocapsid (NC) protein alone were found to bind specifically to an HIV-1 riboprobe. Either Cys-His box of NC could be removed without eliminating specific binding to the Ψ riboprobe, but portions of *gag* containing only the MA and CA proteins without NC did not bind to RNA. There were at least two binding sites in HIV-1 genomic RNA that bound to the *gag* polyprotein: one entirely 5′ to *gag* and one entirely within *gag*. The HIV-1 NC protein bound to riboprobes containing other retroviral Ψ sequences almost as well as to the HIV-1 Ψ riboprobe.

Retroviral RNA encapsidation is the process whereby retrovirus particles acquire two copies of the single-stranded viral genomic RNA. The genomic RNAs dimerize at a region near the 5′ end of the genome (7, 9, 15, 47) and are packaged inside a capsid initially composed of *gag* and *gag-pol* precursor polyproteins. As virions bud from the cell, the *gag* and *gag-pol* polyproteins are cleaved by the retroviral protease (PR) to generate the *gag* matrix (MA), capsid (CA), and nucleocapsid (NC) proteins, the *pol* reverse transcriptase (RT) and integrase (IN) enzymes, and other viral proteins. The nucleocapsid proteins have been shown to bind nonspecifically to the genomic RNAs, effectively coating each RNA with approximately 2,000 NC proteins (17). Upon infection of a new target cell, the RNAs are reverse transcribed into a double-stranded DNA provirus which integrates into the host genome and serves as a template for the synthesis of new genomic RNA species. The nascent genomic RNA can serve as *gag* and *gag-pol* mRNA, or it can be spliced to form envelope mRNA or other mRNAs, or it can be encapsidated by the next generation of retroviral particles.

RNA encapsidation by the virion specifically selects for viral genomic RNA, since it is the most abundant RNA in the virion but makes up less than 1% of the RNA in the cytoplasm of the infected cell. The mechanism behind the selective encapsidation of retroviral genomic RNA is unknown; however, one likely event is the specific recognition of the RNA by the *gag* polyprotein (14, 44, 54, 61). Many experiments have focussed on the identification of the regions in the RNA and polyprotein that bind to each other. In many retroviruses the packaging element in the RNA, often termed Ψ or E, has been shown to reside in the 5′ end of the genome, often extending into the *gag* coding region (1, 3–6, 11, 21, 28, 34, 35, 38, 48, 49, 56, 65, 66). In human immunodeficiency virus type 1 (HIV-1), deletions in the genome between the major splice donor and the *gag* coding region have impaired encapsidation of the genomic RNA (2, 13, 36). Furthermore, in a northwestern in vitro binding assay, segments of HIV-1 RNA within the *gag* coding region were shown to bind to the HIV-1 *gag* polyprotein (37).

The portion of the *gag* polyprotein that is involved in specific binding to Ψ, however, is controversial. In several retroviruses, certain amino acids within the NC region of the *gag* polyprotein have been shown to be required for efficient encapsidation of retroviral genomic RNA in vivo. The NC proteins of all retroviruses (except the spumaviruses) have one or two copies of a conserved, zinc finger-like domain called the Cys-His box which coordinates one zinc ion (8, 22, 26, 27, 45, 51, 58, 59, 62) and folds into a stable, ordered structure (26, 27, 43, 45, 57, 58, 62, 63). Deletion or point mutations in the Cys-His boxes have been shown to diminish packaging of genomic RNA in vivo (2, 19, 24, 25, 31, 40–42). Thus, the NC region of the *gag* polyprotein appears to be necessary for RNA encapsidation. However, a recent report on Rous sarcoma virus has shown that efficient RNA encapsidation can occur when both Cys-His boxes have been deleted from the *gag* polyprotein (4). In addition, although in vitro binding studies have consistently demonstrated that NC proteins bind to single-stranded nucleic acids (12, 16, 18, 30, 32, 39, 52, 55, 64), most studies have failed to identify sequence-specific binding (16, 39, 52, 55, 64). Recently, a gel mobility shift assay has been used to measure in vitro binding of purified bovine leukemia virus (BLV) *gag* proteins to BLV genomic RNA segments; in this report, NC was shown to bind to all RNA segments analyzed, whereas MA was shown to bind only to the RNA segment containing the BLV packaging element Ψ (33). This and other reports (4, 20, 44) indicate that specific RNA encapsidation by the *gag* polyprotein may involve domains other than the NC region.

In this study, we used a gel mobility shift assay to measure binding of wild-type and mutant HIV-1 *gag* polyproteins to segments of the HIV-1 genomic RNA and heterologous RNAs. The proteins were expressed as fusion proteins with glutathione *S*-transferase (GST) and were purified from bacterial extracts; the RNAs were expressed from cloned cDNAs in vitro. We found that both NC and the polyprotein bind with

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specificity to RNAs containing the putative Ψ region. In addition, we show that HIV-1 MA and CA did not bind to RNA in these assays. Furthermore, we show that two separate RNA segments bound with specificity to the *gag* polyprotein: one entirely 5′ to the *gag* coding region and one entirely within the *gag* coding region. Last, we show that HIV-1 NC and *gag* polyproteins bound to other retroviral packaging elements, though not as selectively as to HIV-1 Ψ, indicating that the mechanism of RNA encapsidation may be conserved among the retroviruses.

MATERIALS AND METHODS

Preparation of fusion proteins. Retrovirus nucleotide sequence numbering is with respect to the 5′ edge of the 5′ long terminal repeat of the DNA provirus. *gag* proteins were expressed in bacteria as fusion proteins with GST. The HIV-1 Hxb2 *Nde*I (nucleotide [nt] 789)-*Bcl*I (nt 2428) fragment, containing the entire *gag* gene plus the 5′ end of the *pol* gene, was isolated from plasmid pT7HG (37) and treated with mung bean nuclease to generate blunt ends. The fragment was then ligated to a pGex plasmid (Pharmacia) which had been digested with *Eco*RI and *Bam*HI and also treated with mung bean nuclease. The fusion protein encoded by the resulting plasmid, pGST-GAG, contains the entire *gag* polyprotein except for the N-terminal methionine. Plasmid pGST-NC, encoding the entire NC region of the *gag* polyprotein plus the five residues C terminal to NC, was generated in the same way as plasmid pGST-GAG, except that the Hxb2 *Mnl*I (nt 1919)-*Bgl*II (nt 2095) fragment was used and pGex2TK was linearized only with *Bam*HI.

Plasmids containing deletions in the *gag* polyprotein were constructed from plasmid pGST-GAG by cleaving the plasmid DNA at two unique sites, generating blunt ends with the Klenow fragment of DNA polymerase I, and recircularizing the large fragment with T4 DNA ligase. Plasmid pGST-GAGd1 had a deletion of the region from the *Mun*I site at nt 1967 to the *Avr*II site at nt 2010; the deletion replaces a sequence overlapping the first Cys-His box (CGKEGHTARN CRAP) with a single serine residue. Plasmid pGST-GAGd2 had a deletion of the region from the *Avr*II site at nt 2010 to the *Bgl*II site at nt 2095; this deletion removes a sequence (KKGCWKCGKEGHQMKDCTERQANFLGK) encompassing the second box. Plasmid pGST-NCd1, lacking sequences from nt 1967 to nt 2010, was generated from the NC parent similarly to pGST-GAGd1. Plasmids pGST-GAGNsi and pGST-GAGMun were constructed by linearizing plasmid pGST-GAG at unique sites (*Nsi*I at nt 1250 or *Mun*I at nt 1967) in the *gag* gene, blunting the ends with the Klenow fragment, and recircularizing. As a result, the reading frame is changed at the enzyme site; in pGST-GAGNsi, a glycine and an ochre stop codon were inserted into the 5′ end of CA, and in pGST-GAGMun, an ochre stop codon was inserted into the first Cys-His box of the NC region.

To prepare fusion proteins, expression was induced in *Escherichia coli* DH1 by the addition of 0.8 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to log-phase bacterial cultures for 3 to 4 h. Bacteria were collected by centrifugation, rinsed with phosphate-buffered saline, and lysed in L buffer (20 mM Tris-HCl [pH 8], 100 mM NaCl, 0.5% Nonidet P-40) plus 0.2 mM phenylmethylsulfonyl fluoride. Lysed bacteria were sonicated briefly and cleared of insoluble material by centrifugation. Fusion proteins were collected from the supernatants with glutathione-agarose beads (Sigma) at a concentration of 20 μl of packed beads per ml of bacterial culture. After 1 to 4 h of gentle shaking at 4°C, the beads were collected by centrifugation and rinsed three times with L buffer. Fusion proteins were eluted from the beads by gentle shaking in 50 mM Tris-HCl, pH 8–5 mM reduced glutathione for 1 h at 4°C. Fusion protein yields were estimated by Bradford spectrophotometric analysis. Finally, glycerol was added to the fusion protein preparations to a final concentration of 20% and aliquots were stored at −70°C.

Preparation of riboprobes. Riboprobes were transcribed in vitro from cloned cDNAs. Plasmid pBGPVR, containing the 5′ end of the HIV-1 Hxb2 genome (from the *Bgl*II site at nt 473 to the *Pvu*II site at nt 1146) in the vector pSP72 (Promega), has been described before (previously named pHRBP) (37). Plasmid pMPR, containing the 5′ end of the Mason-Pfizer monkey virus genome, was constructed by subcloning the *Sph*I (nt 171)-*Sac*I (nt 1167) fragment of plasmid pSHRM15 (provided by Sung Rhee, University of Alabama) into the pSP72 polylinker. Plasmid pBLVR, containing the 5′ end of the BLV genome (from the *Sac*I site at nt 357 to the *Sal*I site at nt 1147) was provided by Iyoko Katoh (plasmid pAMG) (33). Plasmid pBACR, containing the 5′ end of the human β-actin cDNA, was constructed in two steps. First, the 2.0-kb *Pst*I-*Bam*HI fragment containing the entire cDNA was subcloned from plasmid pHFβA-1 (provided by Bruce Patterson, National Institutes of Health) into pSP72, generating plasmid pBAC; then the 3′ 1.8 kb of the cDNA was deleted by digesting plasmid pBAC with *Ava*I and recircularizing the large fragment.

Deletions in plasmid pBGPVR between the *Bgl*II site (nt 473) and various 3′ sites were constructed to generate plasmids containing HIV-1 sequences with different 5′ ends. These plasmids include pBSPVR (with a deletion to the *Bss*HII site at nt 708), pCLPVR (with a deletion to *Cla*I at nt 830), and pACPVR (with a deletion to *Acc*I at nt 959). Plasmid pNDPVR was constructed by first introducing an *Nde*I site at the *gag* translation initiator codon of pBGPVR (nt 789) (37) and then deleting sequences between the *Bgl*II site at nt 473 and the *Nde*I site at nt 789.

To synthesize riboprobes, plasmids were first linearized with appropriate restriction enzymes. Plasmid pMPR was linearized with *Spe*I (at nt 989), plasmid pBLVR was linearized with *Dra*I (at nt 746), and plasmid pBACR was linearized with *Eco*RI (in polylinker). Plasmids containing HIV-1 sequences were linearized at any one of several sites: *Bss*HII at nt 708; *Nde*I at nt 789; *Cla*I at nt 830; *Acc*I at nt 959; and *Pvu*II at nt 1144. Riboprobes were synthesized in 20-μl reaction mixtures containing 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine, 10 mM dithiothreitol, 0.5 mM ATP, 0.5 mM UTP, 0.5 mM GTP, 12 μM CTP, 40 U of RNase inhibitor (Boehringer Mannheim), 1 to 2 μg of linearized plasmid DNA, 40 to 50 μCi of [α-^{32}P]CTP (New England Nuclear), and 20 U of either SP6 (pMPR, pBLVR, and pBACR) or T7 (HIV-1 plasmids) RNA polymerase for 1 h at 37°C. After treatment with 10 U of DNase I for 15 min at 37°C, the reaction mixtures were then extracted twice with phenol-chloroform and precipitated with ethanol twice. Finally, the riboprobes were suspended in 50 μl of water and diluted to 20,000 cpm/μl in GS buffer (5 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 2 mM $MgCl_2$, 100 mM KCl, 3.75% glycerol, 20 mM dithiothreitol). All riboprobe preparations were assessed by electrophoresis on a polyacrylamide gel to ensure that the RNAs were intact and that recovery was as expected.

Gel mobility shift assay. Riboprobes were mixed with purified proteins in the following order: 7 μl of GS buffer, 1 μl of yeast tRNA (1 μg), 1 μl of protein (1 pmol), and 1 μl of riboprobe (75 pg). The mixtures were incubated at 30°C for 10

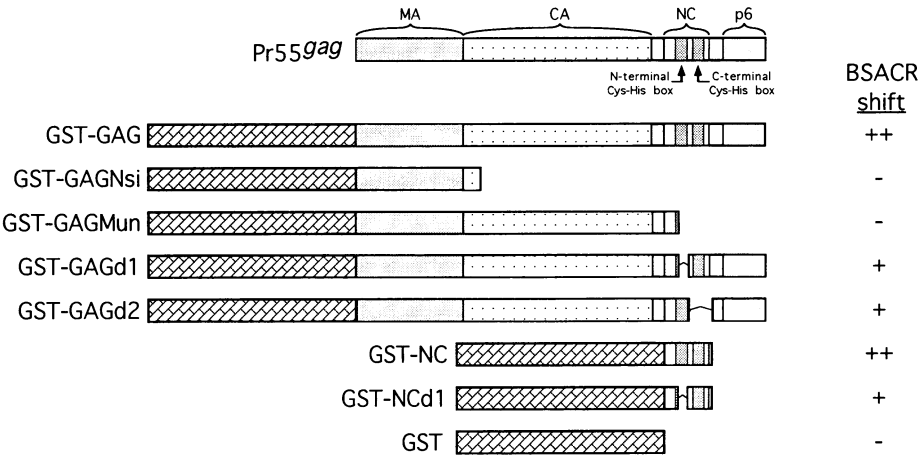

FIG. 1. Schematic of GST-HIV-1 *gag* fusion proteins and their RNA binding activities. (Top) Schematic of wild-type HIV-1 Pr55^{gag} gene product, with matrix (MA), capsid (CA), nucleocapsid (NC), and *gag* p6 regions indicated. The structures of various fusion proteins are indicated below, aligned with the HIV-1 *gag* protein. In all cases, the GST region is at the N terminus. The ability of the fusion proteins to shift an HIV-1 RNA probe (BSACR) is indicated at the right. ++, shifts all the probe under standard conditions; +, shifts approximately half the probe; −, shifts none of the probe.

min, after which 2 μl of 50% glycerol was added to each mixture. The mixtures were then separated by electrophoresis on a polyacrylamide gel (5% acrylamide, 0.5× TBE [45 mM Tris-OH, 45 mM boric acid, 1 mM EDTA], 10% glycerol) in 0.5× TBE for 4 h at 150 V at 4°C; subsequently, the gel was dried and exposed to X-ray film.

Western immunoblot analysis. Fusion proteins in denaturing or native gels were transferred to nitrocellulose filters, and the filters were immersed in TBS buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl) containing 5% milk for 1 h. The filter was then immersed in TBS containing 0.5% milk and either 10 μl of anti-HIV-1 MA monoclonal antibody (DuPont; NEA9282) plus 10 μl of anti-HIV-1 CA monoclonal antibody (DuPont; NEA9283) for denaturing gels or 2 μl of anti-GST rabbit serum for native gels, for 1 h. After rinsing the filter in TBS plus 5% milk, the filter was labelled with biotinylated anti-mouse immunoglobulin (denaturing gels) or biotinylated anti-rabbit immunoglobulin (native gels) antibody for 1 h in TBS and stained with avidin-coupled alkaline phosphatase plus substrate (Vector Laboratories).

RESULTS

Synthesis of GST-NC and GST-GAG fusion proteins. To prepare large quantities of *gag* proteins, various portions of the HIV-1 *gag* gene were fused to sequences encoding the GST protein on bacterial expression plasmids. Two constructs served as parental DNAs for generation of mutants. The protein encoded by pGST-GAG contains the entire *gag* polyprotein precursor, and that encoded by pGST-NC contains only the NC domain with a short C-terminal tail (see Materials and Methods) (Fig. 1).

Bacteria containing either of the two parental DNAs or control plasmid DNA were grown in the presence of IPTG to induce expression of the fusion proteins, and extracts were prepared. The GST fusion proteins were collected by binding to glutathione-coupled agarose beads and then eluted with reduced glutathione. Samples of these preparations were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie to assess the yield and purity of the proteins (Fig. 2). pGST-GAG produced a protein of a mass near the predicted 84 kDa, along with a series of smaller proteins very similar to those identified previously as C-terminal truncations (37). Previous characterization of these proteins suggests that the three major species all

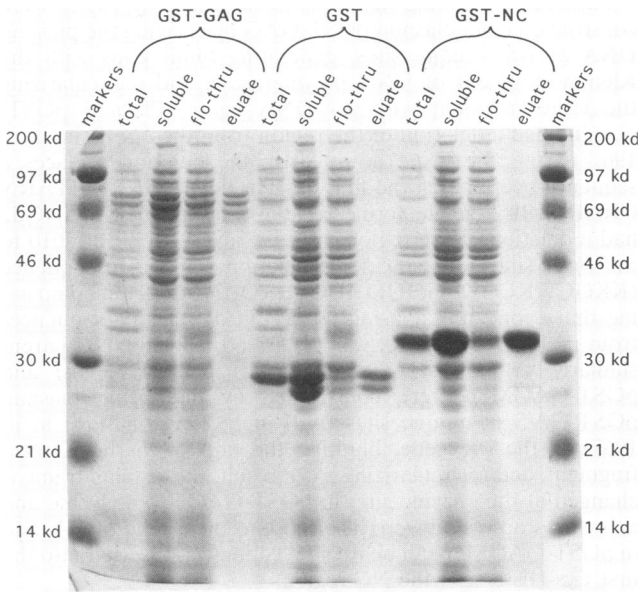

FIG. 2. SDS-PAGE analysis of GST, GST-NC, and GST-GAG fusion proteins. Extracts of bacteria expressing the indicated fusion proteins were prepared, and proteins in various fractions were separated by electrophoresis and visualized by staining with Coomassie brilliant blue. Lanes: total, cells were lysed in SDS-PAGE sample buffer; soluble, cells were lysed with Nonidet P-40 and sonication, and the extract was clarified by high-speed centrifugation; flo-thru, soluble proteins were exposed to glutathione-coupled agarose beads, and the unbound proteins were recovered; eluate, soluble proteins were exposed to glutathione-coupled agarose beads, and the bound proteins were washed and eluted. Aliquots of the four fractions were normalized for the volume of bacterial culture before analysis. The positions of migration of molecular size markers are indicated at the sides.

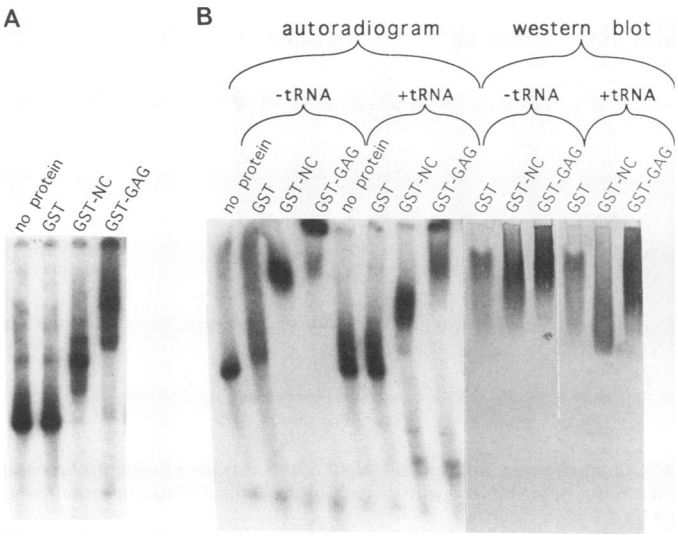

FIG. 3. Standard RNA gel mobility shift assay. (A) The labelled HIV-1 Ψ riboprobe BSACR was mixed with 1 pmol of the indicated fusion protein in the presence of 1 μg of tRNA and subjected to electrophoresis on a native polyacrylamide gel followed by autoradiography. (B) The BSACR riboprobe was mixed with 1 pmol of the indicated fusion protein in the presence or absence of 1 μg of tRNA and subjected to electrophoresis. The gel was either exposed for autoradiography or subjected to Western blot analysis, using antiserum specific for GST. The signals on the autoradiogram represent the BSACR riboprobe; the signals on the Western blot represent the fusion proteins.

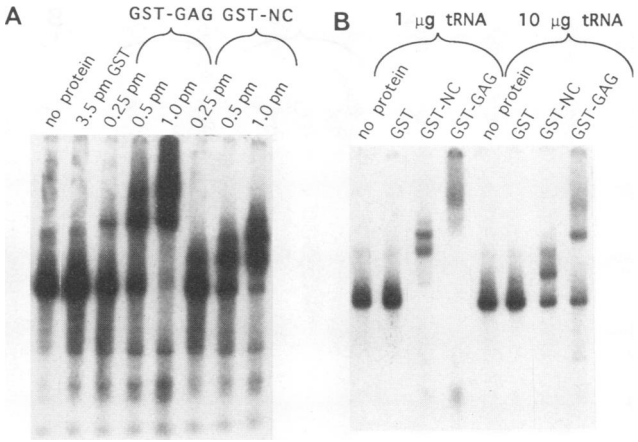

FIG. 4. Effects of decreasing the amount of fusion proteins or increasing the amount of tRNA in the RNA gel mobility shift assay. (A) Seventy-five picograms of the BSACR riboprobe was mixed with different amounts of the indicated fusion protein (in pmol) in the presence of 1 μg of tRNA. (B) Seventy-five picograms of the BSACR riboprobe was mixed with 1 pmol of the indicated fusion protein in the presence of 1 or 10 μg of tRNA.

retain the NC domain, and by northwestern analysis all three retain their ability to bind HIV-1 RNA (37). pGST-NC produced only a single protein of approximately 36 kDa, and the control plasmid produced a doublet of GST proteins of approximately 29 kDa. In each case, the fusion proteins were visible as major proteins in the total lysates; after recovery from the agarose beads, we estimate that the fusion proteins constituted greater than 95% of the total protein in the preparations.

Binding of GST-GAG and GST-NC to an HIV-1 RNA containing the putative Ψ region. To test for binding of fusion proteins to RNA, a radioactive RNA riboprobe from the 5′ end of the HIV-1 Hxb2 genome was synthesized in vitro from cloned cDNAs and mixed with the purified fusion proteins before electrophoresis on a native polyacrylamide gel and autoradiography. A 250-nt riboprobe, termed BSACR, contained sequences beginning approximately 35 nt upstream of the major splice donor and ending approximately 170 nt into the *gag* coding region, including sequences previously shown to be necessary for in vivo packaging of HIV-1 genomic RNA (2, 13, 36) and for binding to the *gag* polyprotein in vitro (37). Binding of 1 pmol of GST-GAG or GST-NC proteins, but not GST alone, to the labelled BSACR riboprobe (75 pg; approximately 1 fmol) was readily detected as a shift in the mobility of the riboprobe in the gel (Fig. 3A). The binding was observed even in the presence of a vast excess of yeast tRNA added as a nonspecific competitor (1 μg or approximately 60 pmol). GST-GAG caused the riboprobe to migrate as a diffuse, low-mobility band, whereas GST-NC shifted the probe to a diffuse band of intermediate mobility, often resolved into a pattern of several discrete bands. In the case of GST-GAG, we cannot determine which of the three major protein species contributed to the binding activity.

Western blot analysis of these native gels indicated that all the proteins migrated as diffuse bands and that the bound riboprobe comigrated with the lower-mobility portions of the GST-NC and GST-GAG bands (Fig. 3B). In contrast, the riboprobe migrated separately from the position of the GST protein. If tRNA was not included in the mixture, the fusion proteins migrated more slowly, indicating that these proteins were binding to the tRNA during the assay. This nonspecific binding is consistent with earlier work demonstrating that both *gag* polyproteins and nucleocapsid proteins bind nonspecifically to RNA in vitro (12, 16, 18, 30, 32, 39, 46, 52, 55, 64).

Reducing the amount of fusion proteins in these experiments resulted in a gradual reduction in the amount of the shifted RNA species (Fig. 4A). As the GST-GAG protein was reduced, the amount of RNA bound dropped very rapidly; the very slowly migrating species were lost first, so that at low protein concentrations only a single species was visible. The nonlinear response to protein concentration suggests that a multimeric form of the protein may be required for binding. As the GST-NC protein was reduced, the amount of RNA bound dropped more linearly with protein. There was a gradual change in the ratios of the various shifted species detected, with only the fastest-migrating species retained at the lowest protein concentrations. These results suggest that the slower forms in the array of bands contain multiple GST-NC protein monomers bound to RNA.

The pattern and extent of binding of the riboprobe to the GST-GAG and GST-NC proteins could also be affected by increasing the amount of tRNA in the mixture. When the amount of tRNA was increased from 1 to 10 μg, only 50% of the riboprobe migrated in the shifted positions; the slowest forms were the most severely affected (Fig. 4B).

Under a variety of conditions, the requirements for binding by GST-NC and GST-GAG were very similar. The binding of the two fusion proteins to RNA could be distinguished, however, by their differential sensitivities to detergents and salt concentrations. The addition of 0.05% SDS to the mixture prevented nearly all of the GST-NC, but not the GST-GAG protein, from binding to the riboprobe (Fig. 5A). Decreasing the amount of monovalent cations, in the absence of divalent cations, prevented GST-GAG, but not GST-NC, from binding

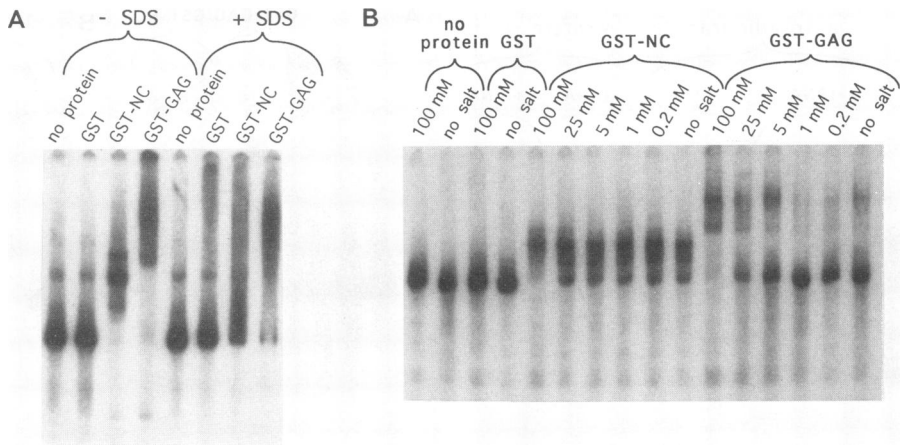

FIG. 5. Effect of SDS and cations on the binding assay. (A) The BSACR riboprobe was mixed with 1 pmol of the indicated fusion protein and 1 μg of tRNA in the presence or absence of 0.05% SDS. (B) The BSACR riboprobe was mixed with 1 pmol of the indicated fusion protein and 1 μg of tRNA in the indicated concentrations of KCl, in the absence of $MgCl_2$.

to the riboprobe (Fig. 5B). Increasing the amount of monovalent cations, either potassium or sodium, up to a total concentration of 250 mM had no effect on binding (data not shown). There was no significant change in the overall binding of either fusion protein when the $MgCl_2$ concentration was increased to 10 mM or when excess EDTA was present.

Specificity of RNA binding exhibited by GST-GAG and GST-NC. To test the sequence specificity of the RNA binding exhibited by the fusion proteins, different riboprobes were synthesized and tested in the mobility shift assay. Each probe was of comparable specific activity, and equal masses of RNA were used in each binding reaction. In contrast to the HIV-1 BSACR riboprobe, virtually none of a radioactive riboprobe corresponding to the 5′ end of the human β-actin mRNA was shifted by either GST-GAG or GST-NC proteins (Fig. 6A). When the amount of tRNA was raised to 10 μg, none of the actin riboprobe appeared to migrate at the shifted positions.

To examine the specificity of binding further, competition experiments were performed, with the addition of unlabelled RNAs to the reactions. Using the BSACR probe, we set up gel shift assays with the standard amount of labelled RNA, competitor tRNA, and the GST-GAG and GST-NC proteins. Addition of 15 or 150 ng of unlabelled actin RNA had little effect on the amount of probe shifted by either protein (Fig. 6B). In contrast, addition of 15 ng of unlabelled BSACR RNA reduced the amount of bound probe somewhat, and addition of 150 ng of this unlabelled RNA almost completely eliminated binding of the probe. It should be noted that 150 ng of this RNA corresponds to about 2 molar equivalents of the protein in these reactions, suggesting that the unlabelled RNA is titrating out the protein. These results suggest that there is indeed a specific recognition of the HIV probe by these proteins.

Some aspects of the RNA structure recognized by the HIV-1 *gag* proteins might be conserved across viral species. We used riboprobes from the 5′ ends of two other retrovirus genomes—BLV and Mason-Pfizer monkey virus—to test for binding by the HIV proteins and observed a shift in the migration of an intermediate amount of these probes (Fig. 6C). In the presence of 1 μg of tRNA, the fusion proteins eliminated almost all of the unbound form of the two riboprobes. The GST-GAG protein moved the RNAs to a diffuse pattern, and it is possible that some of this is attributable to RNA degradation. The GST-NC protein, however, clearly shifted these RNAs to a discrete band. In the presence of 10 μg of tRNA, these riboprobes did not shift but migrated in the same position as with GST alone. These results taken together suggest that the fusion proteins could recognize the labelled HIV-1 sequences well in the presence of competitor tRNA, bound other retroviral Ψ-containing sequences at an intermediate level with low levels of competitor tRNA, and barely bound nonviral RNA sequences at all. Remarkably, the GST-NC protein, containing only the NC domain of *gag*, showed a specificity for HIV-1 RNA comparable to that shown by the GST-GAG fusion protein, which contained the entire *gag* precursor. This result suggests that the rest of *gag* may contribute very little to the specificity of RNA binding during virion assembly.

Determination of the regions in GST-GAG and GST-NC required for binding to HIV-1 RNA. To determine the regions in GST-GAG and GST-NC proteins required for binding to the HIV-1 riboprobes, we constructed deletion and truncation mutations in the *gag* coding region of each expression plasmid (Fig. 1). A deletion spanning part of the N-terminal Cys-His box was introduced into pGST-GAG (pGST-GAGd1) and pGST-NC (pGST-NCd1), and another deletion spanning the C-terminal Cys-His box was introduced into pGST-GAG (pGST-GAGd2). In addition, ochre stop codons were introduced either into the N-terminal domain of the CA region (pGST-GAGNsi) or into the N-terminal Cys-His box (pGST-GAGMun) of pGST-GAG. Mutant proteins were expressed and purified as before; in each case the major product was of the expected size, but there were also several smaller products presumably arising by proteolytic breakdown of the full-length product (Fig. 7). These preparations were assessed for the ability to bind to the HIV-1 BSACR riboprobe and the β-actin riboprobe (Fig. 8). The three deletion mutants, each retaining a single Cys-His box, could bind to the HIV riboprobe approximately 30 to 60% as well as wild-type GST-GAG and GST-NC and retained their sequence specificity, showing very little binding to the actin probe. The two truncation mutants lacking the Cys-His boxes could not bind at all to either riboprobe. Therefore, it appears that under these conditions, either Cys-His box alone can mediate a significant level of specific binding. The deletion of the C-terminal box appears to be more debilitating than the deletion of the N-terminal box, but since the deletions also remove residues outside of the

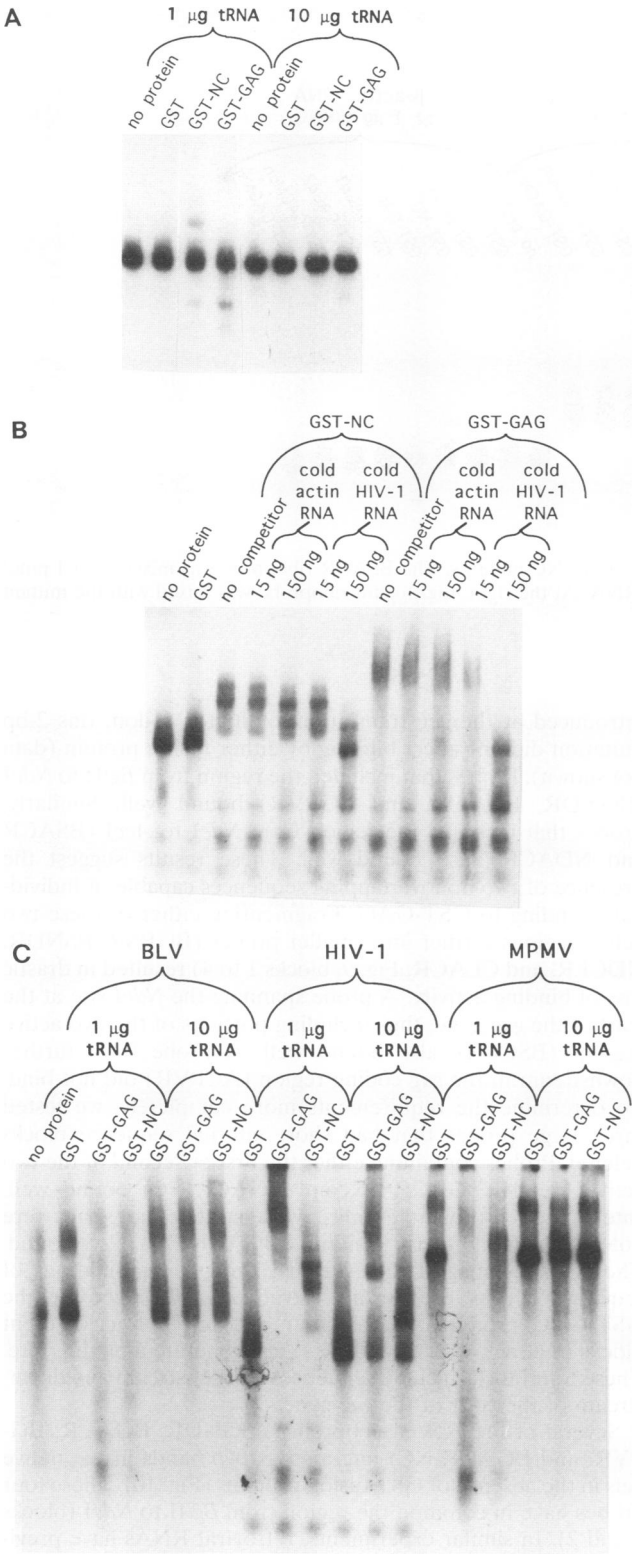

FIG. 6. Specificity of RNA binding by the GST fusion proteins. (A) One picomole of the indicated fusion protein was mixed with a β-actin riboprobe in the presence of 1 or 10 μg of tRNA. (B) Competition of labelled probe by unlabelled RNAs. One picomole of the indicated fusion protein was mixed with the BSACR riboprobe, 1 μg of tRNA, and the indicated amount of unlabelled competitor RNA. (C) One picomole of the indicated fusion protein was mixed with a BLV, Mason-Pfizer monkey virus (MPMV), or HIV-1 (BSACR) Ψ-containing riboprobe in the presence of 1 or 10 μg of tRNA.

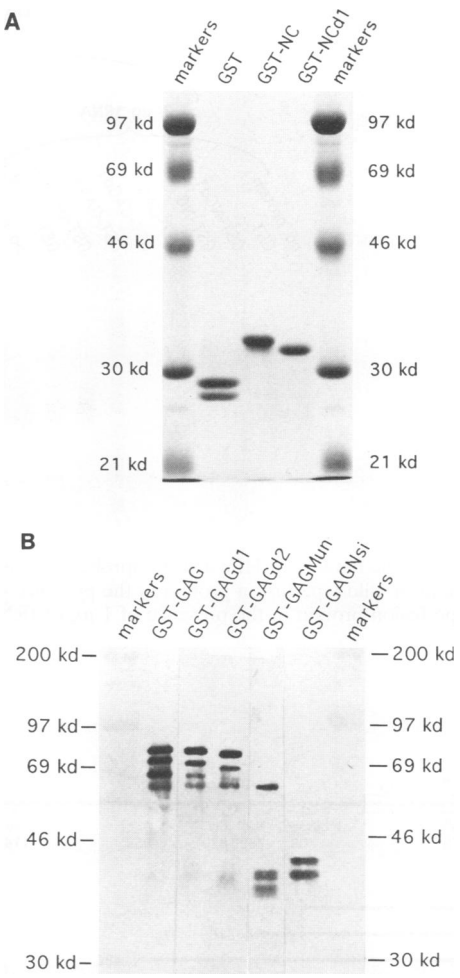

FIG. 7. Electrophoretic mobility of various mutant fusion proteins. (A) Eight micrograms of purified GST, GST-NC, and GST-NCd1 fusion proteins was subjected to SDS-PAGE and Coomassie brilliant blue staining. (B) Ten nanograms of purified GST-GAG, GST-GAGd1, GST-GAGd2, GST-GAGMun, and GST-GAGNsi fusion proteins was subjected to SDS-PAGE and Western blotting, using a mixture of two monoclonal antibodies specific for HIV-1 MA and CA proteins.

boxes, we cannot conclude that this difference is due solely to the differences in sequence within the two boxes.

Determination of binding sites in HIV-1 RNA. To localize the RNA sequences required for recognition by the fusion proteins, different HIV-1 RNA riboprobes were generated and tested for the ability to bind to GST-GAG in the gel shift assay (Fig. 9 and 10). The riboprobes extended between restriction sites and included various sequences from near the 5′ end of the viral genomic RNA through the region encoding the MA portion of the *gag* protein. An *Nde*I restriction site was

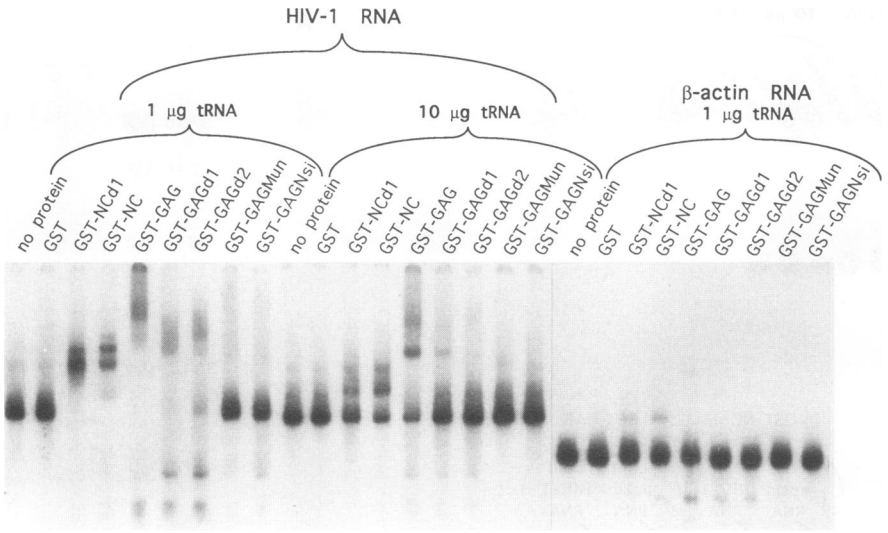

FIG. 8. Specific binding of HIV-1 Ψ riboprobe by various GST-GAG and GST-NC mutants. The BSACR riboprobe was mixed with 1 pmol of the mutant or wild-type fusion protein in the presence of 1 or 10 μg of tRNA. At the right, the β-actin riboprobe was mixed with the mutant or wild-type fusion protein in the presence of 1 μg of tRNA.

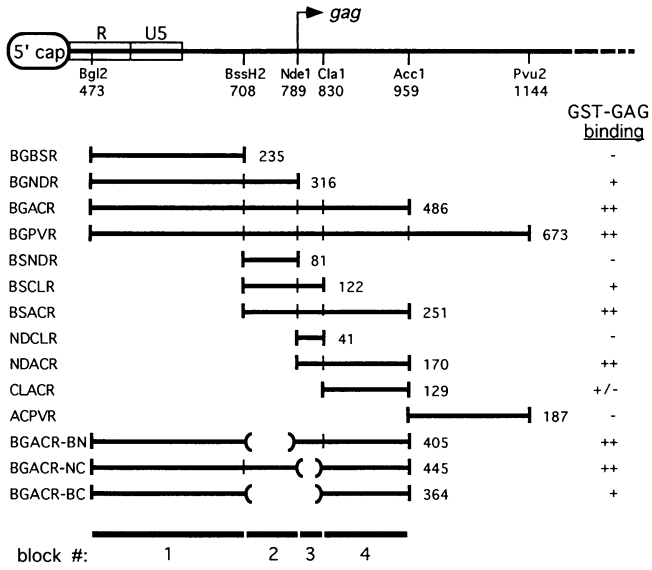

FIG. 9. Schematic of the structures of various HIV-1 riboprobes and their abilities to bind to GST-GAG. (Top) Structure of the 5′ end of the HIV-1 RNA genome. The 5′ cap, R and U5 regions, and start of the *gag* coding region are indicated. Sites for cleavage by various restriction enzymes are indicated, along with their distances in nucleotides from the 5′ edge of the 5′ long terminal repeat of the proviral form. Numbers to the right of each probe indicate its length in nucleotides. Gaps in the RNAs are indicated by open parentheses. (Bottom) Blocks 1 to 4, as delimited by various restriction sites, are indicated (see text). Binding of each RNA is indicated at the right. ++, 100% of the riboprobe bound in the presence of 1 μg of tRNA and at least 25% bound with 10 μg of tRNA; +, at least 80% bound with 1 μg of tRNA and at least 10% bound with 10 μg of tRNA; +/−, at least 60% bound with 1 μg of tRNA but no binding was detected with 10 μg of tRNA; −, less than 10% bound with 1 μg of tRNA.

introduced at the *gag* translational initiator codon; this 2-bp mutation did not affect binding by either fusion protein (data not shown). Probes that included the region from *Bgl*II to *Nde*I (BGNDR, BGACR, and BGPVR) bound well. Similarly, probes that included the region from *Nde*I to *Acc*I (BSACR and NDACR) also bound well. These results suggest the presence of two nonoverlapping sequences capable of individually binding to GST-GAG. Fragmenting either of these two active regions further into smaller probes (BGBSR, BSNDR, NDCLR, and CLACR; Fig. 9, blocks 1 to 4) resulted in drastic loss of binding activity. A probe spanning the *Nde*I site at the start of the *gag* gene, thus including portions of the two active regions (BSCLR), also bound well. A probe from further downstream in the *gag* coding region (ACPVR) did not bind. To determine the requirements more completely, we tested three large probes that had short internal sequence blocks deleted. Probes containing either the first or second of the two active blocks (BGACR-BN and BGACR-NC) bound well. Interestingly, a probe that links two separate regions that were not sufficient alone for binding (BGACR-BC) also bound. These results suggest that various pairwise combinations of sequence blocks can form a structure recognized by the GST-GAG protein. At least four pairings of four different blocks (1+2, 3+4, 2+3, and 1+4) can generate a binding site. These four blocks include sequences both upstream and downstream of the start of the *gag* gene.

Several of the HIV-1 riboprobes (BGNDR, BGACR, BGPVR, and BGACR-NC) migrated as two bands in the native gels in the absence of GST fusion proteins (Fig. 10). These four probes have in common the region from *Bgl*II to *Nde*I (blocks 1 and 2). In similar experiments, retroviral RNAs have previously been shown to spontaneously dimerize in vitro (9, 15, 47). The slower forms detected here may also represent dimeric RNAs. Previous studies have indicated that NC proteins can catalyze the dimerization of retroviral RNA in vitro (9, 15, 47); however, we did not see any evidence for this. Addition of GST-NC to these riboprobes caused shifts in both of the initial bands to two new positions (Fig. 10).

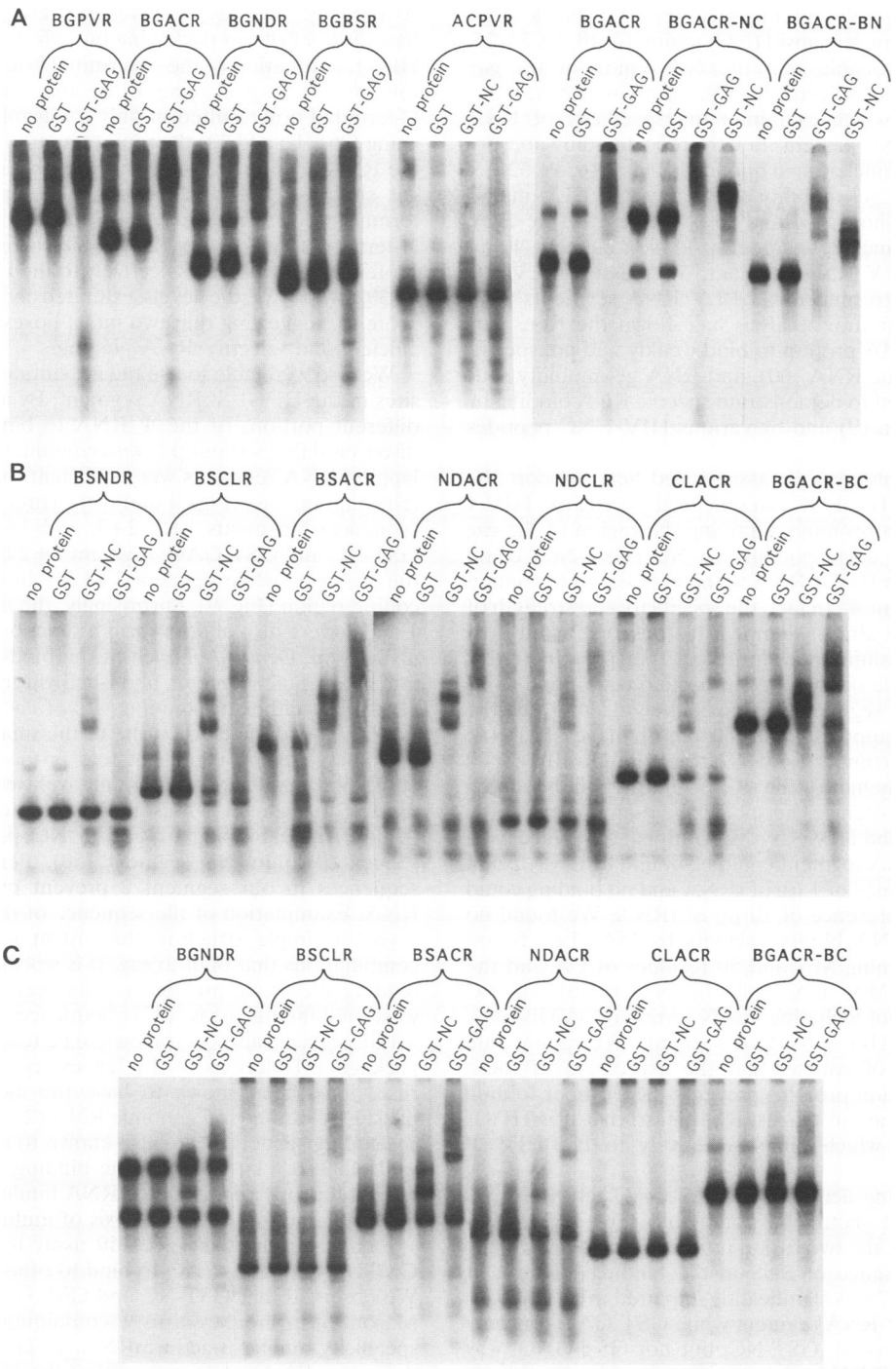

FIG. 10. Ability of the GST fusion proteins to bind different HIV-1 riboprobes. (A and B) Seventy-five picograms of each of the indicated HIV-1 riboprobes was mixed with 1 pmol of the indicated fusion protein in the presence of 1 μg of tRNA. (C) Seventy-five picograms of each of the indicated riboprobes was mixed with 1 pmol of the indicated fusion protein in the presence of 10 μg of tRNA.

DISCUSSION

One important issue in the process of retroviral RNA encapsidation is the identification of the portions of the *gag* polyprotein that are required for specific binding of the genomic RNA. It is very likely that the uncleaved *gag* polyprotein binds to the genomic RNA: protease-deficient virus particles package normal levels of genomic RNA (14, 44, 61); and RNA encapsidation was shown to be normal in particles formed by expression of *gag* alone, without *gag-pol* (44, 54). In addition, the *gag* precursor protein has been shown to exhibit specific RNA binding in vitro (37). Since most, but not all,

mutations of the *gag* polyprotein which impair or block RNA packaging lie in the nucleocapsid (NC) region (2, 10, 19, 23–25, 29, 40–42), it is probable that the NC region of the *gag* polyprotein itself binds selectively to the genomic RNA. However, most studies which examined the specificity of RNA binding by purified NC proteins in vitro reported only nonspecific single-stranded nucleic acid binding activity (16, 39, 52, 55, 64). Using an RNA gel mobility shift assay, the BLV matrix (MA) protein was shown to bind to a segment of the BLV genomic RNA that includes the encapsidation element Ψ but not to any other BLV RNA segment, whereas the BLV NC protein was shown to bind to all BLV RNA segments (33). However, cosedimentation analysis has shown the Rous sarcoma virus (RSV) MA protein to bind weakly and nonspecifically to RSV genomic RNA (60), and RNA gel mobility shift assays have been used to demonstrate specific RNA binding by the RSV NC protein (9) and by various HIV-1 NC peptides (50).

The RNA gel mobility shift assays used here support the conclusion that the HIV-1 NC can specifically recognize HIV-1 RNA. A GST fusion protein containing the region of the *gag* polyprotein roughly corresponding to NC (GST-NC) could bind selectively to an HIV-1 RNA segment believed to contain the packaging element Ψ. In fact, the specificity and strength of the interaction were almost identical to those exhibited by a fusion protein containing the entire *gag* polyprotein (GST-GAG). One picomole of each fusion protein could bind to 75 pg of the Ψ RNA segment (approximately 1 fmol) in the presence of 1 μg (approximately 60 pmol) of yeast tRNA; when the amount of competitor tRNA was raised to 10 μg (600 pmol), approximately half of the Ψ RNAs were still bound by each of the fusion proteins. The specific binding was efficiently inhibited by unlabelled HIV RNA. In contrast, only traces of a human β-actin mRNA segment could be bound by these fusion proteins in the presence of 1 μg of tRNA and no binding could be detected in the presence of 10 μg of tRNA. We found no evidence for any RNA binding activity by MA. Two fusion proteins, one containing MA and 50 residues of CA and the second containing MA, CA, and the N terminus of NC (leading up to, but not including, the N-terminal Cys-His box), did not bind to the HIV-1 Ψ RNA segment. We cannot rule out the possibility, of course, that the expression of these regions of *gag* as fusion proteins prevents their proper folding and binding. It appears in this assay, at least, that it is HIV-1 NC, and not MA, which binds selectively to the HIV Ψ element.

Though the binding activities of GST-GAG and GST-NC were very similar, it should be noted that some differences were observed. First, the two fusion proteins were differentially affected by the presence of SDS in the binding mixture; at 0.05% SDS, GST-NC was significantly impaired in its ability to bind to the HIV-1 Ψ RNA segment while GST-GAG was only slightly impaired. Second, GST-NC, but not GST-GAG, was able to bind to the Ψ RNA when cations were removed from the binding mixture. These differences in behavior may be due to physicochemical differences between the two proteins or to the possibility that GST-GAG, but not GST-NC, may multimerize in the binding mixture.

What is the structure in the NC region of the *gag* polyprotein that binds with specificity to the retroviral genomic RNA? Since most of the mutations in NC which block RNA packaging in vivo lie in the Cys-His box(es) (2, 19, 24, 25, 40, 41), the Cys-His box may form this essential structure. In one previous study with RSV, deletion of either Cys-His box resulted in a partial (10-fold) reduction in RNA packaging while deletions of both boxes resulted in a total block in RNA packaging (41). A more recent study, however, suggests that these mutations have little effect on packaging but affect RNA stability (4). In HIV-1, a mutation of the N-terminal box resulted in an almost total block in packaging while the same mutation in the C-terminal box resulted in only a fourfold reduction in packaging (25). To address the requirement for each Cys-His box in the RNA binding exhibited by GST-GAG and GST-NC in the gel shift assay, we introduced deletions of either the N-terminal or C-terminal Cys-His box into GST-GAG or of the N-terminal Cys-His box into GST-NC. Each mutant fusion protein could still bind selectively to the HIV Ψ RNA segment at 30 to 60% of the levels exhibited by the wild-type fusion proteins, suggesting that two intact boxes are not required for efficient and selective RNA binding.

We also were able to use the gel shift assay to locate binding sites in the HIV-1 Ψ RNA segment. By assaying the ability of different portions of the Ψ RNA to bind to GST-GAG, we discovered that various pairwise combinations of four nonoverlapping RNA segments were sufficient to form a *gag* polyprotein binding site (Fig. 9 and 10). Three pairs of contiguous sequences (segments 1+2, 2+3, and 3+4) all bound well to GST-NC and GST-GAG; segment 1+2 lies immediately 5′ to the *gag* gene, while segment 3+4 lies in the 5′ end of the *gag* coding region (Fig. 9). Surprisingly, the discontinuous regions 1 and 4 were also able to form a functional binding site when juxtaposed. Division of each of the binding-competent RNAs into individual segments abolished the binding activity, indicating that either the binding site of each binding-competent RNA spanned the division site or the smaller RNAs contained a binding site but could not fold properly in the absence of additional sequences. Although we were unable to detect binding to segment 2 in isolation, another group has recently demonstrated binding of HIV-1 NC peptides to a shorter sequence within this segment (50). Perhaps the additional sequences in our segment 2 prevent proper folding of the RNA. Examination of the sequence of the 5′ leader does not reveal a simple structure that might be formed by all the combinations that bind to *gag*. It is worth noting that multiple binding sites have previously been seen in an in vitro northwestern binding assay (37). Sequences both upstream and within *gag* have also been shown to be functionally important in packaging. Deletions in the leader region of several retroviruses have been shown to have the most severe effects on packaging of the viral genomic RNA (2, 35, 36, 38, 53, 66), but *gag* coding sequences are also known to enhance packaging as well (1, 3, 6, 11). Whether the binding sites defined here in vitro will prove necessary for RNA binding in vivo remains to be determined by direct analysis of mutants in animal cells.

Finally, we used the gel shift assay to assess the ability of GST-NC and GST-GAG to bind to other retroviral Ψ RNAs. We observed that GST-NC and GST-GAG bound to BLV or Mason-Pfizer monkey virus Ψ-containing RNA segments with specificity, unlike β-actin mRNA. In fact, GST-NC bound to these two RNAs almost as well as the HIV-1 Ψ RNA; in the presence of 1 μg of tRNA, all of the BLV and Mason-Pfizer monkey virus RNAs were bound, whereas in the presence of 10 μg of tRNA, less than 20% of these two RNAs was bound. Given that intraspecies and interspecies (49) cross-packaging of retroviral RNAs has been seen in vivo, this result is not entirely surprising. We surmise that all retroviruses may use similar structures in the genomic RNA and *gag* polyprotein to achieve efficient and specific RNA encapsidation.

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